SUPPLEMENTAL DATA

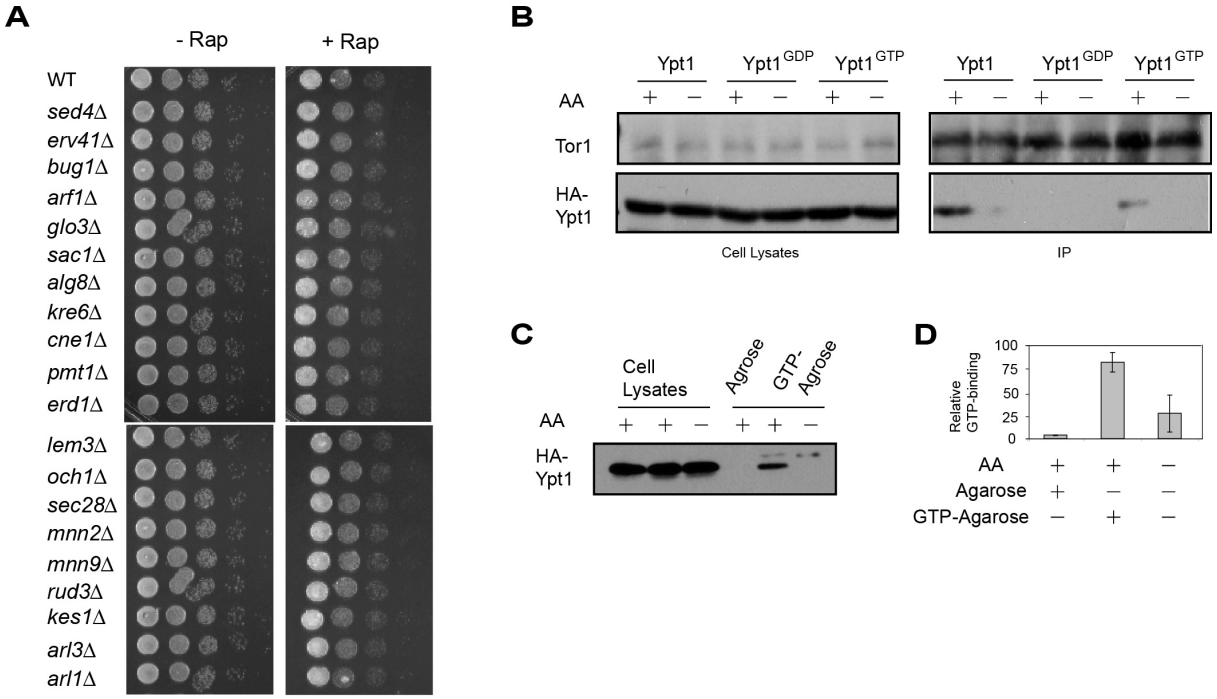


Figure S1, related to Figure 1. **Ypt1 is involved in AA signaling to TORC1 in yeast**

(A) Yeast mutants in the ER-Golgi trafficking pathway were assayed for rapamycin sensitivity. Yeast cells were serially diluted by 10-fold and spotted on YPD plates or YPD plates containing 1 nM rapamycin, and incubated at 30°C for 2 and 5 day, respectively.

(B) Exponentially growing yeast cells expressing HA-Ypt1, HA-Ypt1^{GDP} or HA-Ypt1^{GTP} were starved and re-stimulated with AAs for 1 hr. Tor1 was immunoprecipitated with a Tor1-specific antibody. The binding of HA-Ypt1 to TORC1 was assayed by immunoblot.

(C) Yeast cells expressing HA-Ypt1 were starved and re-simulated with AAs for 1 hr. Cell extracts were incubated with GTP-Agarose beads. GTP-Agarose bound materials were analyzed by Immunoblot. Agarose alone was used as a negative control.

(D) Quantification of the results in Figure S1C. Data represent means ± SD in three independent experiments.

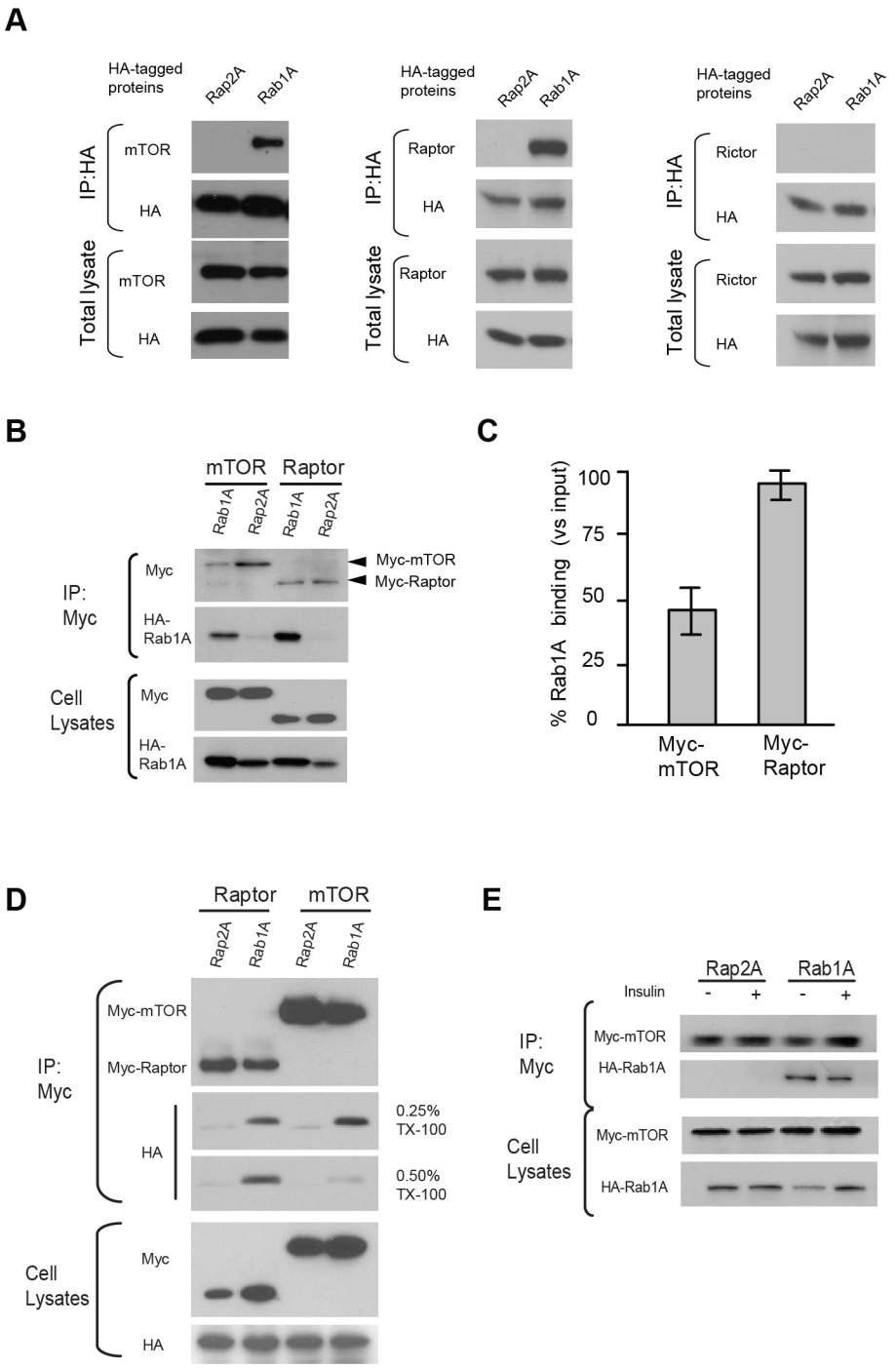


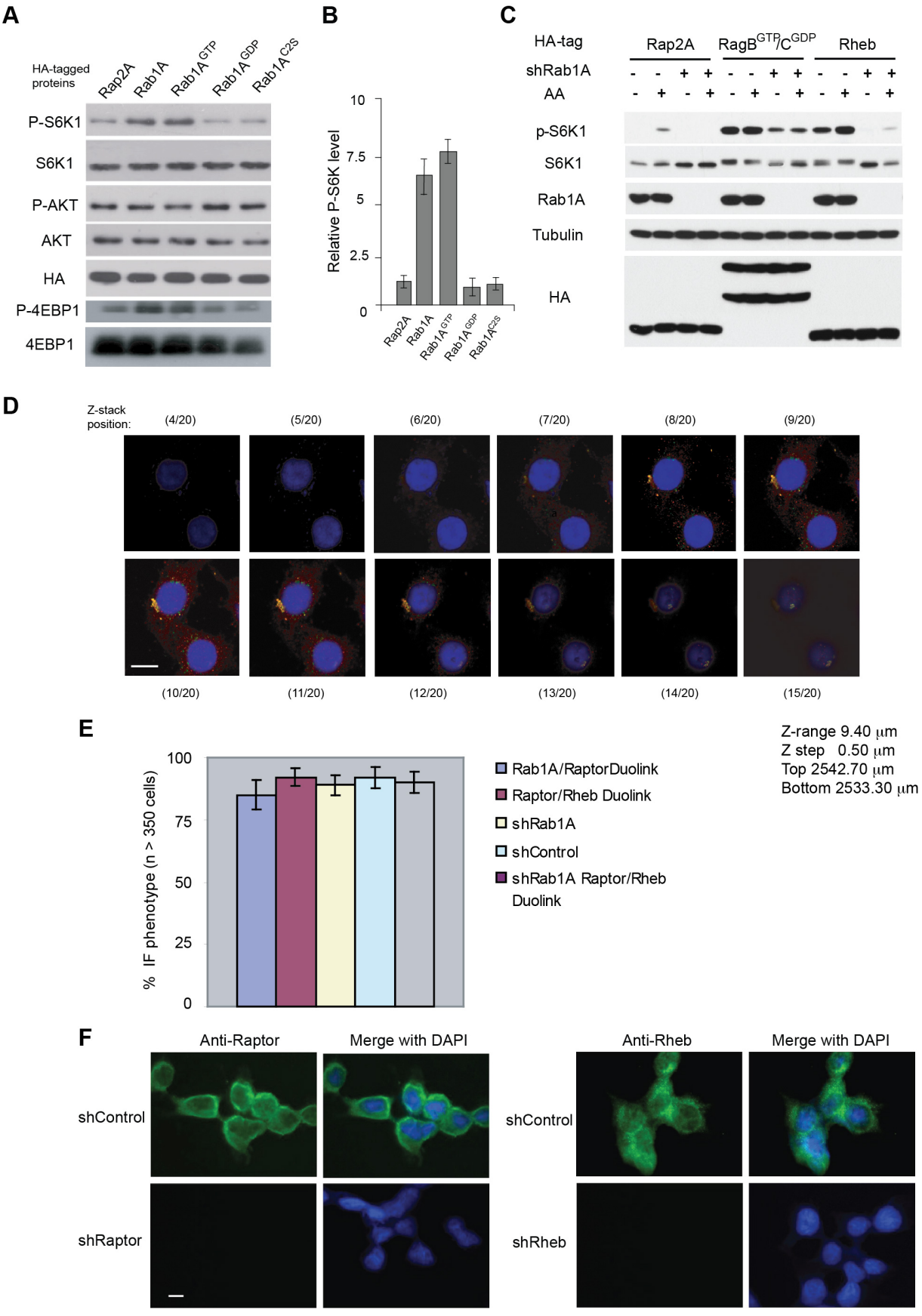
Figure S2, related to Figure 2. Rab1A interaction with mTORC1 and its relationship with Rag and Rheb small GTPases

(A) HA-Rab1A or HA-Rap2A was transiently expressed in HEK-293E cells. HA-tagged proteins were immunoprecipitated and analyzed for their interaction with endogenous mTOR, Raptor or Rictor.

(B) HA-Rab1A was co-expressed with Myc-mTOR or Myc-Raptor in HEK293E cells. Myc-tagged proteins were immunoprecipitated and the binding of HA-Rab1A was determined by immunoblot. HA-Rap2A was used as a negative control.

(C) Quantification of HA-Rab1A bound to Myc-mTOR or Myc-Raptor in (B). Shown is a percentage of the total input. Error bars represent SD.

(D) HA-Rab1A was co-expressed with Myc-mTOR or Myc-Raptor in HEK293E cells. Myc-tagged proteins were immunoprecipitated and washed with buffer containing 0.25% or 0.50% TX-100. The binding of HA-Rab1A was determined by immunoblot.
(E) HEK-293E cells expressing Myc-mTOR and WT or mutant HA-Rab1A proteins were starved from serum and re-stimulated with insulin. HA-tagged proteins were immunoprecipitated and analyzed for their interaction with the endogenous Myc-mTOR. HA-Rap2A was used as a negative control.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HA-tagged proteins
Rap2A
Rab1A
Rab1A GTP
Rab1A GDP
Rab1A C2S
P-S6K1
S6K1
P-AKT
AKT
HA
P-4EBP1
4EBP1
B
Relative P-S6K level
10
7.5
5
2.5
0
Rap2A
Rab1A
Rab1A GTP
Rab1A GDP
Rab1A C2S
C
HA-tag
Rap2A
RagB GTP/C GDP
Rheb
shRab1A
AA
p-S6K1
S6K1
Rab1A
Tubulin
HA
D
Z-stack position:
(4/20)
(5/20)
(6/20)
(7/20)
(8/20)
(9/20)
(10/20)
(11/20)
(12/20)
(13/20)
(14/20)
(15/20)
E
% IF phenotype (n > 350 cells)
100
75
50
25
0
Rab1A/RaptorDuolink
Raptor/Rheb Duolink
shRab1A
shControl
shRab1A Raptor/Rheb Duolink
Z-range 9.40 μm
Z step 0.50 μm
Top 2542.70 μm
Bottom 2533.30 μm
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Merge with DAPI
Anti-Rheb
Merge with DAPI
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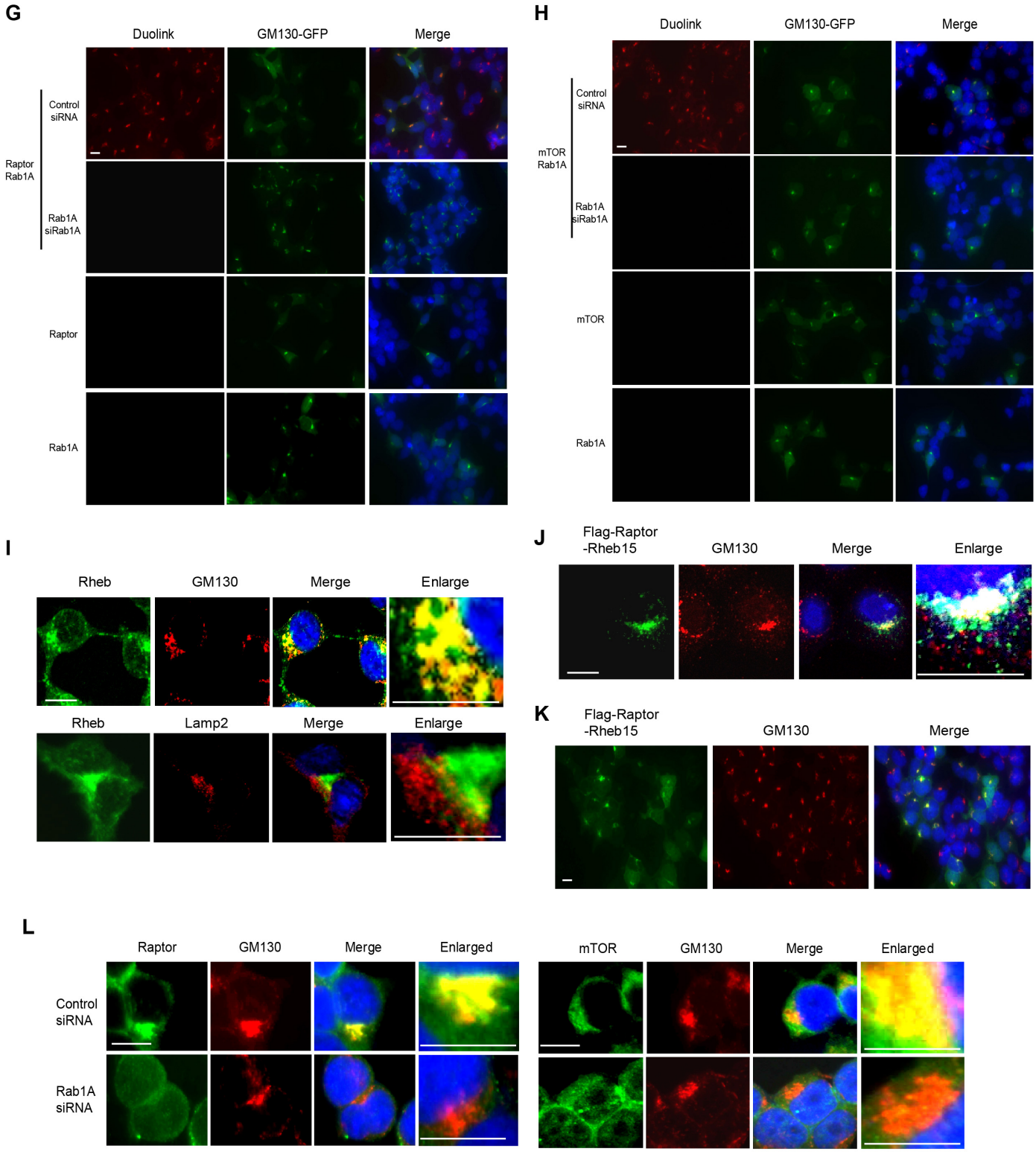
shControl
shRheb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GM130-GFP
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Merge
Control siRNA
mTOR Rab1A
Rab1A siRab1A
mTOR
Rab1A
I
Rheb
GM130
Merge
Enlarge
Rheb
Lamp2
Merge
Enlarge
J
Flag-Raptor -Rheb15
GM130
Merge
Enlarge
K
Flag-Raptor -Rheb15
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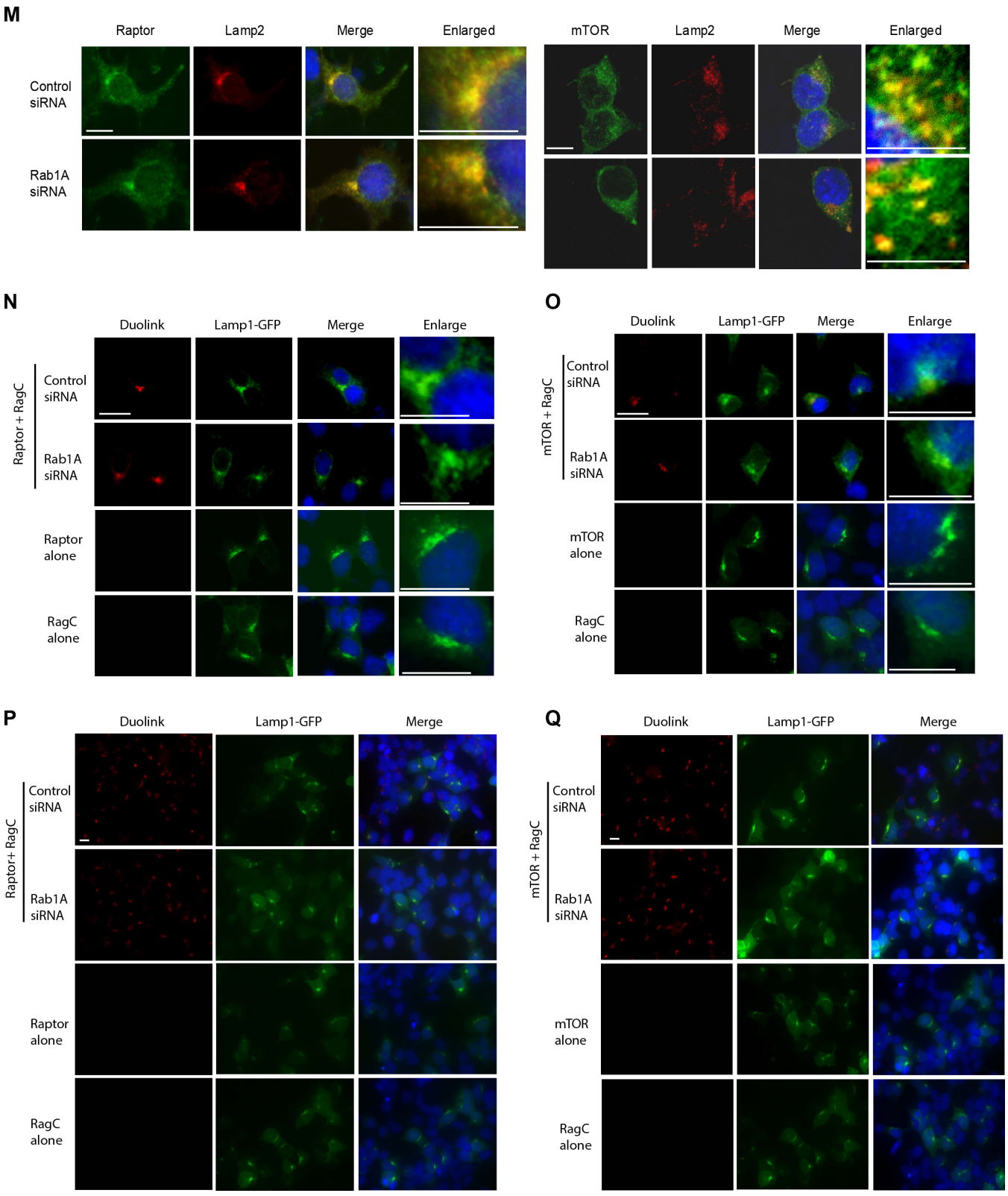
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Raptor
Lamp2
Merge
Enlarged
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Lamp2
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Rab1A siRNA
N
Duolink
Lamp1-GFP
Merge
Enlarge
Raptor + RagC
Control siRNA
Rab1A siRNA
Raptor alone
RagC alone
O
Duolink
Lamp1-GFP
Merge
Enlarge
mTOR + RagC
Control siRNA
Rab1A siRNA
mTOR alone
RagC alone
P
Duolink
Lamp1-GFP
Merge
Raptor+ RagC
Control siRNA
Rab1A siRNA
Raptor alone
RagC alone
Q
Duolink
Lamp1-GFP
Merge
mTOR + RagC
Control siRNA
Rab1A siRNA
mTOR alone
RagC alone

Figure S3, related to Figure 3. Mechanism of mTORC1 regulation by Rab1A in HEK293E cells

(A) HA-Rab1 proteins were transiently expressed in HEK392E cells. The level of P-S6K, S6K1, P-AKT, AKT, P-4EBP1 and 4EBP1 was analyzed by immunoblot. HA-Rap2A was used as a negative control.

(B) Quantification of relative S6K1 phosphorylation (P-S6K1/total S6K1, arbitrary unit). Error bars represent SD.

(C) HEK293E cells infected with Rab1A shRNA or a control shRNA in the presence or absence of overexpressed $RagB^{GTP}/C^{GDP}$ or Rheb were starved with AAs (containing all culture ingredients except AAs) and re-stimulated with AAs for 10 min. The effect of Rab1A knockdown on P-S6K1 was analyzed by immunoblot.

(D) The in situ interaction between Rheb and Raptor (Red) was determined by Duolink in HEK293E cells expressing GM130-GFP (Golgi marker, Green), and analyzed by confocal microscopy. Shown are consecutive Z-sections of merged images that clearly show Rheb-Raptor interaction (Yellow) in the Golgi. Scale bar = 10 μm.

(E) Quantification of Duolink images in Figure 3. Shown is the percentage of cells with described phenotype (n > 350). Error bars represent SD.

(F) HEK293E cells expressing control, Raptor or Rheb1 shRNAs were stained by IF with Raptor or Rheb1 antibodies used in the Duolink study. Scale bar = 10 μm.

(G) The in situ interaction between Rab1A and Raptor (Red) was determined by Duolink in HEK293E cells transiently expressing GM130-GFP (Golgi marker, Green) in the presence of a control siRNA or Rab1A siRNA. Antibody against Rab1A or Raptor alone was used as negative controls. Images were captured by fluorescence microscopy. Shown are low magnification images to provide an overview of the Rab1A-Raptor interaction in the Golgi, which occurs in nearly 100% cells. Scale bar = 10 μm.

(H) Same as Figure S3G except mTOR antibody is used. Shown are low magnification images to provide an overview of the Rab1A-mTOR interaction in the Golgi, which occurs in nearly 100% cells. Scale bar = 10 μm.

(I) HEK293E cells were analyzed for Rheb localization (Green) in the Golgi (GM130, Red) or lysosomes (Lamp2, Red) by confocal immunofluorescence microscopy. The nucleus was stained with DAPI (Blue). Shown are single sections of images representative of over 90% cells. Scale bar = 10 μm.

(J) HEK293E cells transiently expressing Flag-Raptor-Rheb15 were analyzed for Flag-Raptor-Rheb15 (Green) localization in the Golgi (GM130, Red) by confocal immunofluorescence microscopy. The nucleus was stained with DAPI (Blue). Shown are single sections of confocal images representative of over 90% cells. Scale bar = 10 μm.

(K) The same as (J) except images are shown at a low magnification that indicates Flag-Rheb15 is prominently co-localized with the Golgi in nearly 100% cells. Scale bar = 10 μm.

(L) HEK293E cells were transiently transfected with Rab1A or a control siRNA. Raptor and mTOR localization in the Golgi was analyzed by confocal immunofluorescence microscopy using antibodies against endogenous Raptor (Green), mTOR (Green) and GM130 (Red, Golgi marker). The nucleus was stained with DAPI (Blue). Shown are single sections of confocal images representative of over 90% cells. Scale bar = 10 μm.

(M) HEK293E cells were transiently transfected with Rab1A or a control siRNA. Raptor and mTOR localization in the lysosomes was analyzed by confocal immunofluorescence microscopy using antibodies against endogenous Raptor (Green), mTOR (Green) and Lamp2 (Red, lysosomal marker). The nucleus was stained with DAPI (Blue). Shown are single sections of confocal images representative of over 90% cells. Scale bar = 10 μm.

(N) The in situ interaction between RagC and Raptor (Red) was determined by Duolink in HEK293E cells transiently expressing Lamp1-GFP (lysosomal marker, Green) in the presence of a control siRNA

or Rab1A siRNA. Antibody against RagC or Raptor alone was used as negative controls. Images were captured by fluorescence microscopy. Scale bar = 10 μm.
(O) Similar to (N) except mTOR antibody was used. Scale bar = 10 μm.
(P) Same as (N) except shown are low magnification images that contain a large number of cells, indicating that the RagC-Raptor interaction in the lysosomes occurs in nearly all cells. Scale bar = 10 μm.
(Q) Same as (N) except shown are low magnification images that contain a large number of cells, indicating that the RagC-Raptor interaction in the lysosomes occurs in nearly all cells. Scale bar = 10 μm.

Table S1, Related To Figure 4. Correlation between Rab1A level and clinical parameters of CRC cases

Clinicopathological parameters	Cases	Rab1A			p value
		H	L	N	
Age (yr, Median=70, Range=24~84)					0.6193
</=70	47	17	21	9	
>70	43	20	16	7	
Gender					0.9239
Male	47	19	19	9	
Female	43	18	18	7	
Tumor location					0.5700
Right and transverse colon	47	20	17	10	
Left colon	43	17	20	6	
Histological grade					0.6998
1	9	2	5	2	
2	47	20	20	7	
3	34	15	12	7	
Lymph node metastasis					0.0739
No	53	18	27	8	
Yes	37	19	10	8	
Depth of invasion					0.0017
T1+T2	9	3	2	4	
T3	68	26	35	7	
T4	13	7	1	5	
Stage					0.0414
I	7	2	2	3	
II	46	16	25	5	
III+IV	37	19	10	8	

H, high Rab1A-positive staining; L, low Rab1A-positive staining; N, Rab1A-negative staining

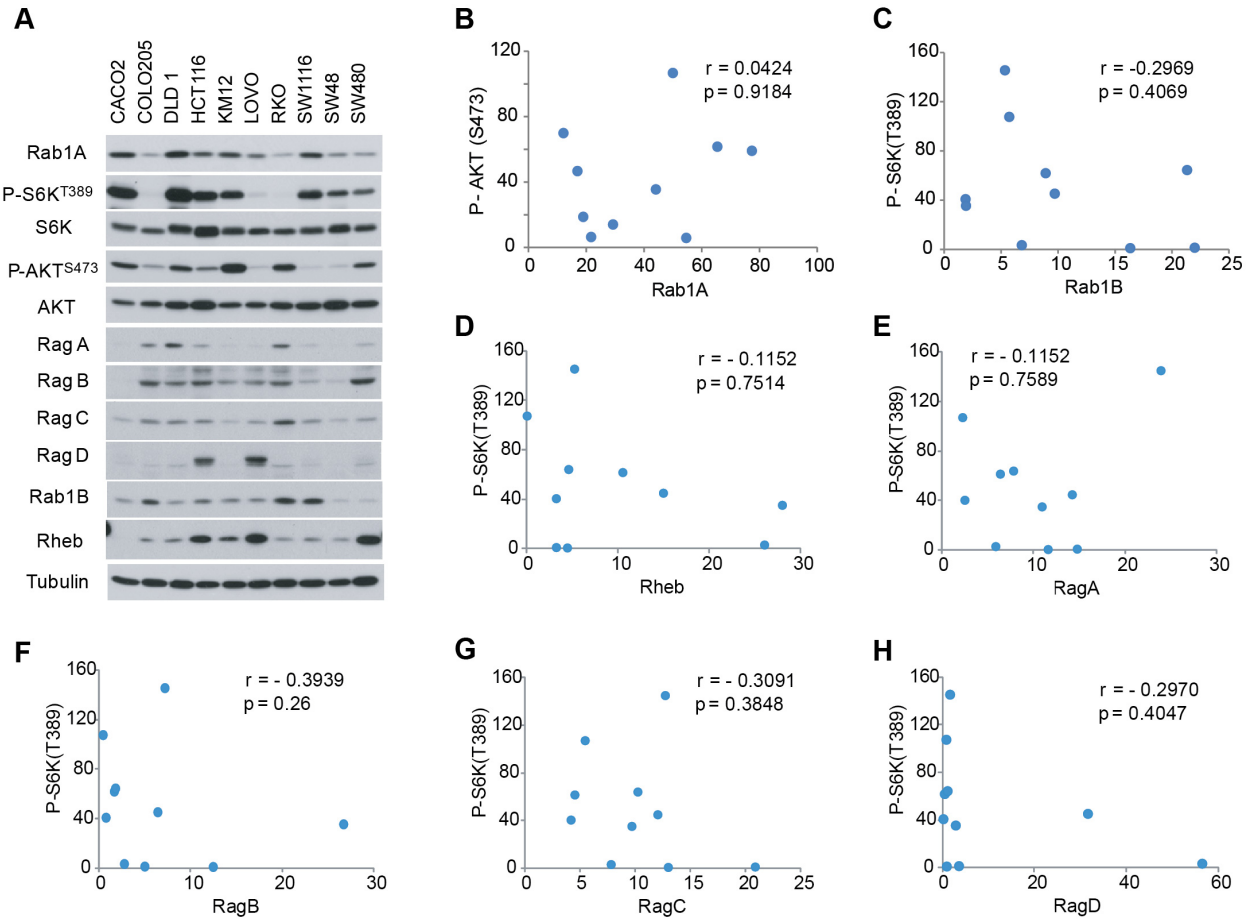

Figure S4, related to Figure 5. Expression of Rab1A, but not of Rab1B, RagA/B/C/D or Rheb, is correlated with mTORC1 signaling in CRC cells

(A) A panel of common CRC cell lines was analyzed for the expression of Rab1A, P-S6K, S6K, P-AKT, AKT, RagA, RagB, RagC, RagD, Rab1B and Rheb by immunoblot.

(B) Correlation plot between Rab1A expression and P-AKT(S473).

(C-H) Correlation between P-S6K1(T389) and the expression of Rab1B, Rheb, RagA, RagB, RagC and RagD, respectively.

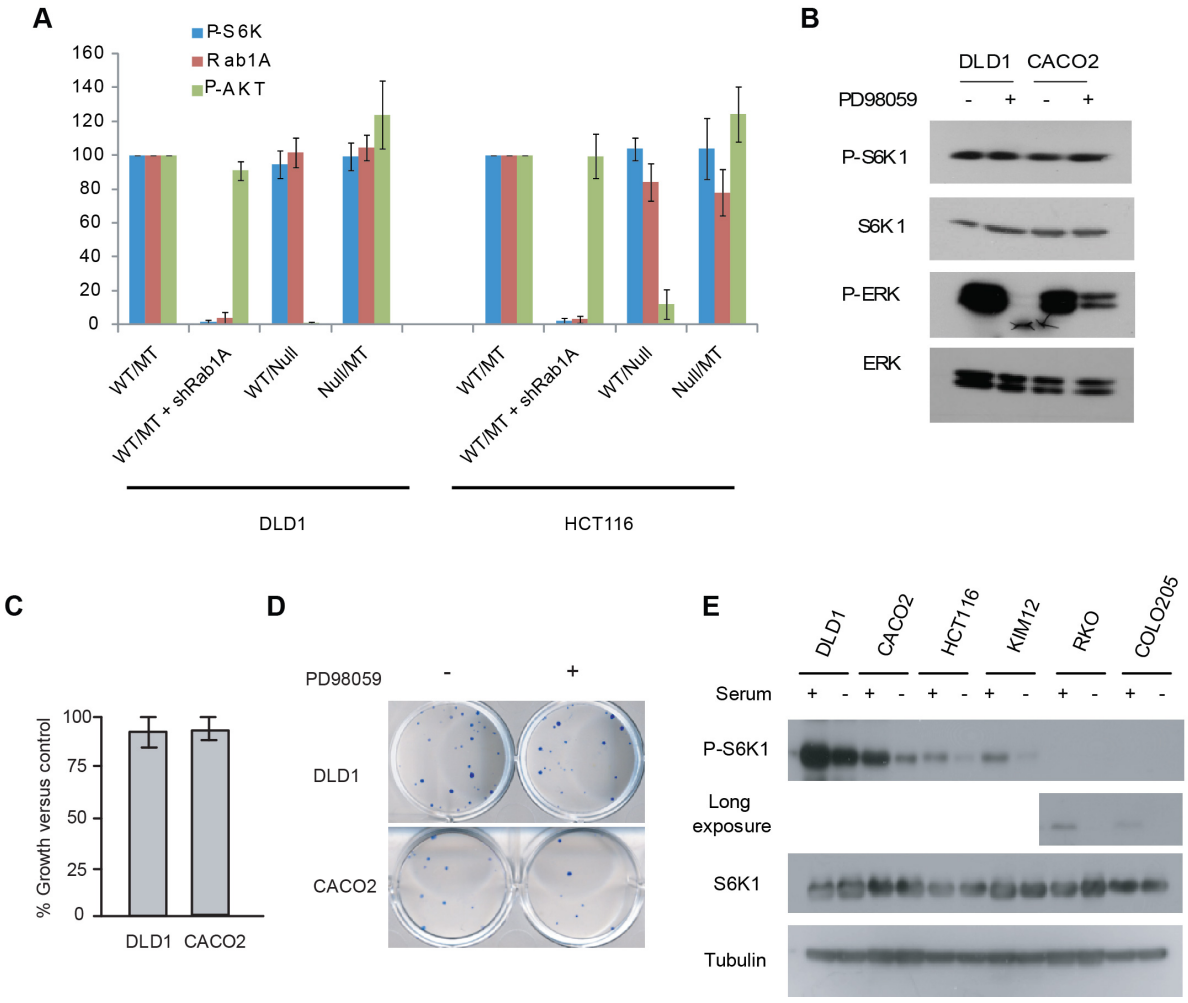

Figure S5, related to Figure 6. Rab1A overexpression but not activation of PI3K and MEK/ERK pathways is crucial for hyperactive mTORC1 signaling and oncogenic growth of CRC

(A) DLD1 and HCT116 cells with Rab1A knockdown, or deletion of WT or mutant PIK3CA allele were analyzed for Rab1A protein expression, and the level of P-S6K1 and P-AKT by immunoblot. The results represent means ± SD from four independent experiments.

(B-D) DLD1 and CACO2 cells were treated with 10 μM PD98059 and the effect on ERK phosphorylation (B), cell growth (C) and colony formation (D) was assayed. The results in C represent means ± SD in three independent triplicate experiments.

(E) CRC cell lines were cultured in complete or serum-free medium for 12 hr. The effect of serum starvation on P-S6K1 was determined by immunoblot.

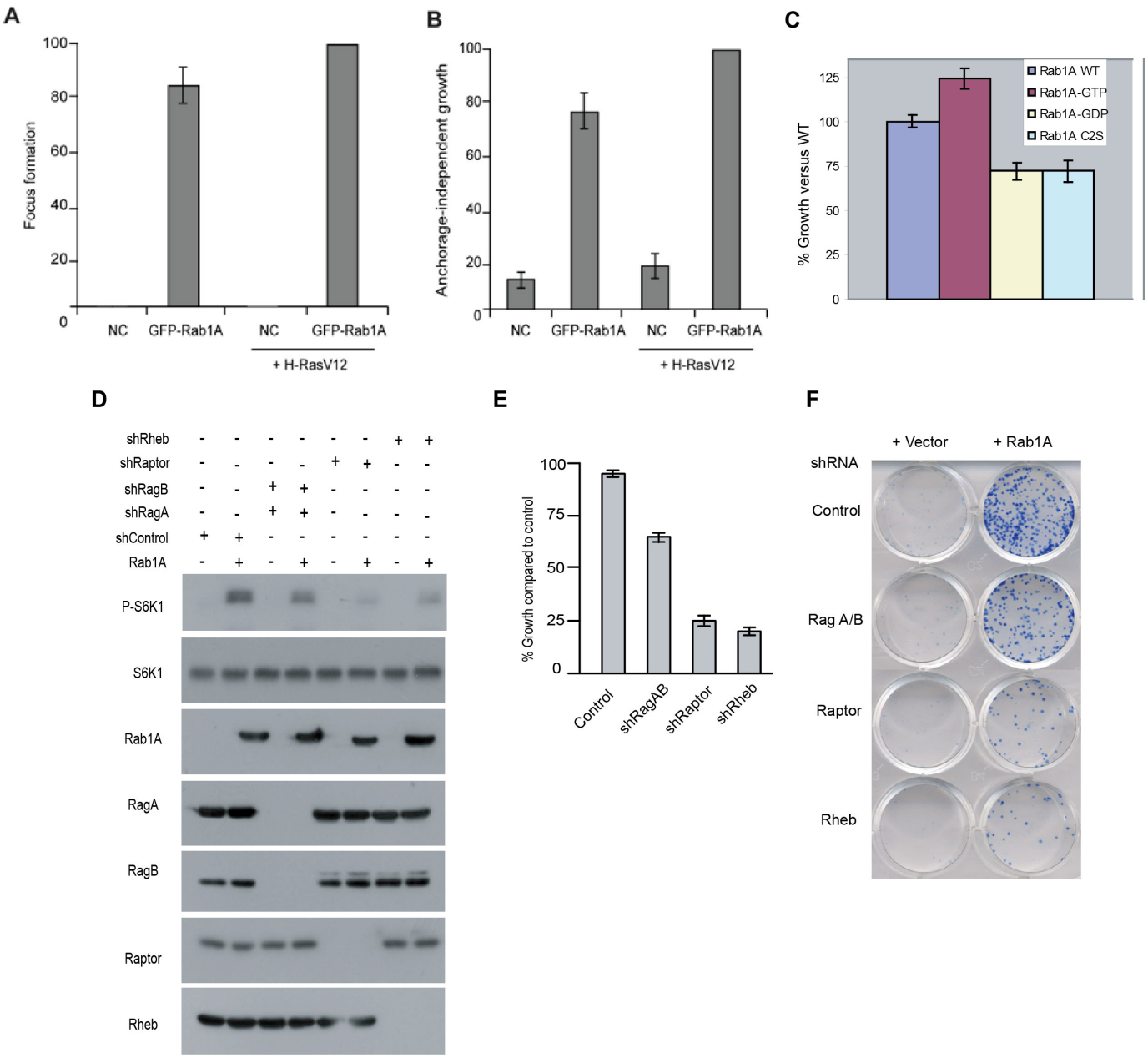

Figure S6, related to Figure 7. Rab1A overexpression promotes focus formation and anchorage-independent growth

(A) Relative focus formation efficiency (arbitrary unit) of confluent NIH3T3 or NIH3T3/H-Ras^{V12} cells stably expressing GFP or GFP-Rab1A. The data represent means ± SD from three independent triplicate experiments.

(B) Relative anchorage-independent growth (arbitrary unit) in soft agar of NIH3T3 or NIH3T3/H-Ras^{V12} cells stably expressing GFP or GFP-Rab1A. Data represent means ± SD from three independent triplicate experiments.

(C) Relative growth of mutant Rab1A versus WT Rab1A. Data represent means ± SD from three

independent triplicate experiments.
(D) RKO cells overexpressing GFP-Rab1A were infected with Lentiviral shRNA against Rheb, Raptor or RagA/B. Their effect on P-S6K1 and S6K was assayed by immunoblot.
(E) RKO cells overexpressing GFP-Rab1A were infected with Lentivirus carrying shRNA against Rheb, Raptor or RagA/B. The effect of shRNAs on cell growth was assayed by SRB assay. Data represent means ± SD from three independent triplicate experiments.
(F) RKO cells with a vector control or overexpressing GFP-Rab1A were infected with Lentivirus carrying shRNA against Rheb, Raptor or RagA/B. The effect of shRNAs was assayed by colony formation assay. Shown are representative images of the colony formation experiment.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Yeast Strains

Wild type (WT) and mutant yeast strains are in the S288C/FM391 genetic background (MATa *hisD1 leu2D0 met15D0 ura3D0*); Yeast deletion strains were obtained from Open Biosystems with corresponding loci replaced by KanMX. Yeast strains with essential genes under the control of tetracycline promoter (Tet-promoter) were also purchased from Open Biosystems. The *ypt1-ts* and isogenic WT strains were kind gifts from Dr. Nava Segev (Richardson et al., 1998). *MAF1-MYC* and *HA-SCH9* plasmids under the control of their native promoters were previously generated (Wei et al., 2009; Wei and Zheng, 2009). Yeast cells were cultured in synthetic complete (SC) medium to early log phase (OD600 =0.4). For amino acid (AA) starvation and re-stimulation, cells were shifted to SC medium without AAs for 1 hr, and then incubated in SC medium without or with AAs for 1 hr. Cells were collected and broken in lysis buffer (50mM Tris-HCl pH7.5, 150mM NaCl, 0.5 mM EDTA, 0.5% NP-40, 2mM PMSF, Roche protease Complete inhibitor cocktail and phosSTOP tablet) with a glass beads beator at 4˚C. Rapamycin sensitivity assay was performed as previously described (Chan et al., 2000).

Mammalian cell lines and cell culture

NIH3T3, HEK-293E and HEK293T cells were cultured in DMEM with 10% fetal bovine serum (FBS). Human colon cancer cell lines (CACO-2, COLO-205, DLD-1, HCT116, KM-12, LOVO, RKO, SW1116 and SW480) were cultured as described previously (Zhang and Zheng, 2012). Isogenic DLD-1 and HCT116 cell lines with deletion of WT and mutant PIK3CA were generous gifts from Dr. Bert Vogelstein and cultured as previously described (Samuels et al., 2005). For plasmid DNA transfection, HEK293E cells were seeded at 2 x 10^6 cells/10 cm dish. Cells were

transfected with plasmid DNA by calcium phosphate (Choi et al., 2002; Tsang et al., 2010). For generation of cell lines stably expressing Rab1A-GFP, HEK293E and the colon cancer cell lines were transfected with Rab1A-GFP using Lipofectamine 2000 (Life Technologies). 48 hr post transfection, G418 (geneticin) was used for selection and after 2 weeks, cells were analyzed for stable Rab1A expression and used for analysis as described.

siRNAs and shRNAs

pGIPZ lentiviral shRNAs were obtained from Thermo Fisher Scientific. The pGIPZ number for each shRNA is: human Rab1A shRNA (V3LHS_175283, V2LHS_220706, V2LHS_173283), human Rheb (V3LHS_637690, V3LHS_637691, V3LHS_637686), human Raptor (V3LHS_636803, V3LHS_200995, V3LHS_636800), human RagA (V3LHS_352383, V3LHS_352384, V2LHS_199517), human RagB (V2LHS_70562, V3LHS_411342, V3LHS_411343), and non-silencing control shRNA (RHS4346). HEK293T cells were transfected with shRNA encoding plasmids with psPAX2 packaging plasmid and pMD2.G envelope plasmid (Addgene; Dr. Didier Trono, EPFL, SV-LVG, Laboratory of Virology and Genetics, Station 15, CH-1015 Lausanne) using calcium phosphatate reagents. Supernatant containing packaged virus was collected 48 hr later, filtered and applied to target cells with 8 μg/ml polybrene. After 24 hr, puromycin was used for selection and cells were analyzed as indicated after the third day post infection. For generation of stable shRNA expressing cells, cell cultures were maintained under puromycin selection for 2 weeks. Rab1A siRNAs (sequences 2,3,4) and Rab1B siRNAs (sequences 2,3,4) were designed as described previously (Haas et al., 2007) and synthesized by Integrated DNA Technologies. For siRNA transfection, cells were

incubated with siRNA oligonucleotides and D-FECT (Dharmacon) for 6 hr. The cell culture was then replenished with fresh culture medium and the cells were analyzed as indicated.

Nutrient starvation and re-stimulation of mammalian cells

Nutrient and serum starvation and re-stimulation experiments were generally carried out as previously described by Sabatini and co-workers (Sancak, et al., 2008). For AA starvation and re-stimulation, almost confluent cells were rinsed once with PBS, and incubated in RPMI-1640 without AAs with high glucose (R1780, Sigma-Aldrich, USA) and 10% dialyzed FBS for 50 min, and re-stimulated with 10x AA mixture in above medium for 10 min and analyzed as indicated. For serum and insulin starvation and stimulation of cells, almost confluent cells were rinsed once in PBS and incubated in complete RPMI without FBS for 12 hr, and stimulated with 10x dialyzed FBS or 100 nM insulin for 10 min in RPMI and analyzed as indicated. For nutrient sensitivity assays, CRC cells at 30% confluence were rinsed with PBS, and then incubated in 1640-RPMI with complete complement of ingredients except different percentage (25%, 50%, 75% or 100%) of AAs, dialyzed FBS, or glucose for 3 day. Cell growth was analyzed as indicated.

Duolink proximal ligation

HEK293E cells were cultured on coverslips, transfected with GM130-GFP plasmid by calcium phosphate and fixed using 4% paraformaldehyde after growth for 24 hr. Cells were blocked with 5% goat serum in phosphate buffered saline with 0.2% Triton X-100. The coverslips were incubated in primary antibodies as indicated for 12 hr at 4°C. Antibodies from different species that recognize two target epitopes were used. A single antibody was used as a negative control.

Duolink® In Situ PLA® Probe Anti-Rabbit PLUS and Duolink® In Situ PLA® Probe Anti-Mouse MINUS with Duolink® In SituDetection Reagents Red (DUO92008, DUO92002, DUO02004, Sigma-Aldrich, USA) were used for amplification and detection of bound PLA (proximity ligation amplification) probes. Duolink assay was preformed following manufacturer's product instruction. For the Rab1A knockdown experiment, HEK293E cells cultured on coverslips were transfected with Rab1A siRNA as described above. After 48 hr, cells were fixed and Duolink assay was performed as described above. All coverslips were mounted on microscope slides using Duolink In Situ Mounting Media with 4',6-diamidino-2-phenylindole (DAPI). Images were acquired using an Olympus fluorescence microscope at a magnification of 100x or a Carl Zeiss LSM700 laser confocal scanning microscope at a magnification of 63x.

GTP-binding assay

For binding of Rab1A to GTP-Agarose beads, cells were harvested and suspended in binding buffer (20 mM HEPES pH 8, 150 nM NaCl, 10 mM $MgCl_2$, 5x Roche Protease Cocktail Inhibitor Complete) and lysed using three freeze thaw cycles. The cell lysates were centrifuged at 14,000x g and the supernatants were incubated with 100 μl of GTP-Agarose suspension (G9768, Sigma Aldrich, USA) for 1 hr with rotation at 4°C. The beads were pelleted by centrifugation, washed three times in binding buffer and suspended in 40 μl SDS-PAGE sample buffer (Rehman et al., 2008). The binding assay for radioactively GTP/GDP was adopted from that for Rag GTPases (Kim et al., 2008; Sancak et al., 2008)**.** Briefly, confluent HEK-293T cells cultured in DMEM with FBS were rinsed once with phosphate-and serum-free DMEM, and then incubated in phosphate-and serum-free DMEM containing 0.5 mCi 32P orthophosphate (Perkin Elmer) for 4 hr. Cells were rinsed with serum-, AA-, and phosphate-free RPMI, incubated with

serum-, AA-, and phosphate-free media containing 0.5 mCi [^{32}P]-orthophosphate for 50 min, and then re-stimulated with AAs as previously described. After rinsing with ice-cold PBS, cells were lysed in 0.5 ml lysis buffer (50 mM HEPES KOH (pH 7.4), 100 mM NaCl, 1 mM KH2PO4, 1 mM ATP, 100 μM GDP, 100 μM GTP, 5 mM MgCl2, 1% Triton X-100), centrifuged for 10 min at 13,000 rpm at 4°C. The supernatant was incubated with 15 μl of Rab1A antibody (Proteintech) for 1 hr at 4°C. 20 μl of protein G beads were added and incubated for another 45 min. The beads were washed 8 times with wash buffer (50 mM HEPES KOH [pH 7.4], 500 mM NaCl, 5 mM MgCl2, 0.5% Triton X-100, 0.005% SDS) and bound nucleotides were eluted in 23 μl of elution buffer (10 mM EDTA, 2 mM DTT, 0.2% SDS, 0.5 mM GDP, 0.5 mM GTP) at 60°C for 10 min. 10 μl samples were spotted on PEI cellulose TLC plates and dried before immersion in methanol then placed in a chamber filled with 1 cm 0.7 M KH_2PO_4 pH 3.4 until the solvent reached the top of the plate. The result was analyzed by a phosphoimager (BioRad Quantity One).

Cell growth, colony formation and anchorage-independent growth

The cell growth, colony formation and soft agar assays were performed as previously described (Zhang and Zheng, 2012). Colony number for colony formation is expressed as the number of colonies per well in 12-well plates. Soft agar data is expressed as number of colonies per well in 6-well plates. Data represent mean ± SD from three independent triplicate experiments.

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